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In vivo chaperone assisted folding of a 1,6-fucosyltransferase from *Rhizobium* sp.

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Experimental Section

Materials: The main characteristic of the plasmids and bacterial strains used in this study are summarized in Table 1. Taq DNA polymerase was purchased from Ecogen (Spain). T4 DNA ligase was obtained from MBI Fermentas AB (Lithuania). Restriction enzymes Eco RI and XhoI were purchased from Boehringer Manheim (Germany). IPTG was purchased from Applichem GmBH (Germany). Kits for purification of plasmids and PCR products were from Promega (Madison, WI). Kit for purification of DNA fragments was from Eppendorf (Hamburg, Germany). Pyruvate Kinase/L-Lactic Dehydrogenase Type II from rabbit muscle, phosphoenol pyruvic acid (PEP), β-NADH, chloramphenicol and L-arabinose were obtained from Sigma (St. Louis, MO). *N,N'*-Diacetylchitobiose was obtained from Toronto Research Chemicals (Ontario, Canada). GDP-β-L-Fucose was purchased from Kyowa Hakko Kogyo Co., Ltd. (Japan).Low molecular

weight calibration kit for SDS-PAGE was from Amersham Pharmacia Biotech. (UK). Nickel-iminodiacetic acid (Ni^{2+}-IDA) agarose was generous donated by Hispanagar S. A. (Spain). The bead size of the gel used was 40-180 μm with a crosslinked agarose concentration of 6%. The Ni^{2+} charged was 20-35 μmol/ml gel. All other chemicals were purchased from commercial sources as reagent grade. UV/Visible spectra were recorded on a Spectra max Plus 384 spectrophotometer at 25 ^{o}C. SDS-PAGE was performed in a Mighty-Small Mini-Vertical Electrophoresis Unit SE-250 (Hoefer Scientific Instruments). GC analysis was carried out on a Hewlett Packard Chromatograph 5890 Series II, with FID detector, using a SPB-1 capillary column (3m, 0.25 mm id, and 0.25 μm film).

Sub-cloning of a 1,6-FucT in pRSET plasmid and cell transformation: DNA manipulation was according to standard procedures.[1] The *nodZ* gene was amplified by PCR using as template the purified plasmid pKK1,6FucT. The oligonucleotides 5′-ATTCT**CTCGAG**ATGTACAATCGATAT-3′ and 5′-ATATA**GAATTC**TCAAGAGGCGGTATT-3′ were used as leftward and rightward primers respectively (the recognition sequence for XhoI and Eco RI are in bold). PCR amplification was performed in a 100 μL reaction mixture containing 0.1 μL of DNA template, 73.5 μL of water, 10 μL of 10 × buffer (100 mM Tris-HCL, 500 mM KCl, pH=8.0), 2 mM $MgCl_2$, 200 μM of

dNTPs, 1 μM of primers and 2.5 U of Taq DNA polymerase. The reaction was subjected to 30 cycles of amplifications. The cycle conditions were set as follows: denaturation at 94 °C for 1 min, annealing at 55 °C for 1.5 min, and elongation at 72 °C for 1 min. The PCR amplification product was purified and double digested with Xho I (100 U) and Eco RI (100 U) prior to be ligated to the double digested pRSET plasmid to give pRSET1,6FucT. This plasmid was transformed alone in the *E. coli* strain BL21(DE3) pLys.

The plasmids pAG harbouring the chaperones GroEL/GroES was used to individually cotransform with the plasmid pKK1,6FucT in the *E. coli* strain XL1 Blue MRF'. When plasmid pAG was used to transform together with pKK1,6FucT, the transformation efficiency was extremely low. We decided to transform firstly with the plasmid bearing the molecular chaperone. Cells were plated on LB agar Petri dishes containing 34 μg/mL chloramphenicol. Five positive colonies were randomly selected and grown up to make them competents. Competents cells were chemically prepared following standard protocols.[1] After that, the cells were transformed with pKK1,6FucT. The same protocol was used to cotransform the pAG plasmid along with pRSET1,6FucT in the *E. coli* strain BL21(DE3).

Expression of the recombinant α1,6-FucT in absence or in presence of molecular chaperones: Plasmid pKK1,6FucT was induced as described previously.[2] BL21(DE3) plys cells

carrying the pRSET1,6FucT plasmid were cultured in LB medium containing ampicillin (250 mg/L) at 37ºC with shaking. When the cell culture reached an O.D. of 0.5, the temperature was switched to 30 ºC and the culture was induced with 1 mM IPTG. The expression level was analyzed by SDS-PAGE using gels with 12.5% of polyacrylamide in the separation zone.[3] For the coexpression of the chaperones, the cells were cultured in LB medium containing 250 mg/L ampicillin and 34 mg/L chloramphenicol, at 37 ºC with shaking. When the cell growth reached an O.D. of 0.6, L-arabinose was added to a final concentration of 50 mg/L to induce the expression of the molecular chaperones. The culture was maintained 30 minutes with shaking and then the temperature was switched to 30 ºC, or 18 ºC when indicated, and the culture was induced with 1 mM IPTG to induce the expression of the α 1,6-FucT. The expression was analysed by SDS-PAGE as described above.

Preparation of cell free extract (CFE): The culture broth was centrifuged (10,000 x g, 10 min, 4 ºC), and the cell pellet treated with Tris buffer (8 mL/g cells; 50mM, pH 8.0) containing EDTA (50 mM, pH 8.2) and lysozyme (2 mg/g cell). The suspension was gently stirred at room temperature during 1 h and then kept at 4 ºC overnight. The preparation was gently sonicated for 40 s and cooled down in ice (4 times) to decrease viscosity. DNase (10 μg/gr cell) and $MgCl_2$ (0.95 μg/mL of preparation) were added, and

the mixture was refrigerated for 20 min. The mixture was then centrifuged for 30 min at 13,000 x g to separate the soluble proteins from the inclusion bodies. Streptomicine sulphate (1%) was added to the supernatant and this kept at 4ºC during 20 minutes. The mixture was then centrifuged for 30 min at 13,000 x g to remove the nucleic acids.

Activity and stability assays: The α 1,6-FucT activity was assayed with a coupled enzymatic system, where the decrease of NADH absorbance at 340 nm is directly proportional to the release of GDP during the fucosyltransferase-catalyzed reaction.[4] The activity was measured at 25 ºC for 20 min in a final volume of 0.3 mL, containing 12 mM Hepes (pH 7.7), 50 mM ClK, 6.5 mM $MgCl_2$, 0.7 mM PEP, 0.2 mM NADH, 6 U pyruvate kinase, 13 mU lactate dehydrogenase, 100 μM of chitobiose and 52 μM of GDP-Fucose. Acceptor was omitted for the blank. The assay was initiated upon addition of 15 μL of the α 1,6-FucT soluble or 10 mg of immobilized enzyme and the decrease in the absorbance at 340 nm was monitored. Protein concentration was determined using the Bio-Rad Protein Assay kit, based on the Bradford dye-binding procedure. One unit of enzyme activity is defined as the amount that catalyzes the transfer of 1 μmol of fucose from GDP-Fuc to chitobiose per min.

For stability studies soluble or immobilized fucosyltransferase was incubated in phosphate buffer (10

mM, pH 7.4) at 4ºC without stirring. At different times aliquots were withdrawn and assayed for activity.

Purification of recombinant a 1,6-FucT by Cibacron Blue 3GA- and Reactive Red 120 agarose: The CFE obtained as described above was treated with ammonium sulfate (20-40%). After dialysis, 2 mL of the enzyme preparation were loaded on a 1 mL column of CB 3GA or RR 120, previously equilibrated with 50mM cacodilate buffer, pH 7.0, containing 40 mM $MgCL_2$, and 10% glycerol. The non specifically-bound proteins were washed with 20mL of the same buffer. The fucosyltransferase was subsequently eluted with a step-gradient of KI (0.25, 0.5 and 1.0 M) in 20 mL of cacodilate buffer, pH 7.0, containing 10% glycerol.

IMAC purification/immobilization of histidine-tagged a 1,6-FucT: 1.5 mL of CFE were applied to 0.5 gr of Ni2+-IDA-agarose, previously equilibrated with 20 mM phosphate buffer, pH 8.0 containing 10 mM imidazole. The non specifically retained proteins were washed with 10 mL of the same buffer. In order to elute the immobilized H-T FucT, the resin was equilibrated with phosphate buffer 20mM, pH 8.0 containing 20% glycerol and 0.1% Triton X-100. Finally, The H-T FucT was eluted with the same buffer containing imidazole (1 M).

Fucosylation of chitobiose catalysed by the immobilized H-T FucT: 0.7 g of Ni^{2+}-IDA-agarose with H-T FucT immobilized (0.2 U/g gel) were added to a solution of chitobiose (4.7

μm, 3.0 mg) and GDP-Fucose (14.0 μm, 6.0 mg) 1.5 mL of Hepes buffer (15 mM pH 7.4) containing $MnCl_2$ (10 mM). Aliquots (0.1 mL) were withdrawn at different times to be analyzed by GC. After filtration, the samples were lyophilized and then dissolved in pyridine (5 μL) containing 1 mM of benzyl β-D-xylopiranoside as internal standard and trimethylsilylimidazole (5 μL). The mixture was heated at 60 ºC for 30 min. GC analysis was carried out with the following temperature program: initial temperature 195ºC during 5 min; rate 15ºC/min; final temperature 260ºC.

References

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Table 1. Bacterial Strains and Plasmids used in this study

Strains	**Relevant characteristics**	**Source**
XL1-Blue MRF′	Δ(mcrA)183 Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F′ proAB $lacl^qZ\Delta M15$ $Tn10(Tet^r)$]	Stratagene Co.
BL21(DE3)	F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm λ (DE3)	Invitrogen
BL21(DE3) pLys	F^- ompT $hsdS_B$ ($r_B^-m_B^-$) gal dcm λ (DE3) pLysS (Cam^R)	Invitrogen
Plasmids		
pKK223-3	pBR322 origin, Amp^r, P_{tac} promoter	Pharmacia Biotech. Inc.
pRSET	F1 origin, Amp^r, T7 promoter, His Tagg/N-t	Invitrogene
pKK1,6FucT	pKK223-3 containing *nod Z* gene under P_{tac} promoter.	ref[2]
pAG	pAR3[5], containig GroEL/ES under *araBpo* promoter-operator, Cam^r.	ref[6]
pRSET1,6FucT	pRSET, containing *nod Z* gene under T7 promoter	This study